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BIOCHEMICAL CHARACTERIZATION OF A SPHINGOMONAD ISOLATE FROM THE ASCOCARP OF WHITE TRUFFLE (*TUBER MAGNATUM* PICO)

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Abstract - Available information on bacteria that influence the economically important white truffle (*Tuber magnatum* Pico*)* life cycle is scarce. From the ascocarp of white truffle we isolated a strain TMG 022C, capable for growth in nitrogen-depleted conditions and assimilation of mannitol and trehalose. According to 16S rDNA sequence phylogeny, the strain was closely related to *Sphingobium amiense*. The strain had the ability to perform ammonification, reduce nitrate and solubilize $Ca_3(PO_4)_2$, produce chitinase, lipase, phospholipase and β-glucanase, but not cellulase, pectinase, protease and siderophores. The results suggest that *Sphingobium sp*. TMG 022C could have an influence on the *Tuber magnatum* life cycle through improved mycelium nutrition and ascocarp decomposition.

Key words: Sphingobium, phosphate solubilization, *Tuber magnatum*

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INTRODUCTION

Bacteria may influence the development of ectomycorrhizal fungi by improving fungal nutrition by enhancing the availability of scarce nutrients (N, P, micronutrients), through nitrogen fixation or chemical transformation, phosphate solubilization, production of siderophores or biocontrol agents (Frey-Klett et al., 2007). In addition, bacteria can have an active role in ascocarp decomposition and spore dispersal. Since the bulk soil is often limited in nutrients available to microorganisms, the selective pressure of mycorrhizal fungi on bacteria may improve multitrophic interactions, where nutrients may become available in the soil around the hyphae. Trehalose and mannitol, the main mobile carbohydrates in ectomycorrhizal fungi (Koide et al., 2000) have been reported to be responsible for the selection of specific bacterial communities in mycorrhizosphere (Rangel-Castro et al., 2002; Uroz et al., 2007). For example, a clear shift in the soil *Sphingomonadaceae* community in the mycosphere of *Russula exalbicans* and *Laccaria proxima* in comparison to the bulk soil community, was recorded by Boersma et al. (2009), indicating the selection of specific sphingomonads by these ectomycorrhizal fungi. Their investigation for the first time revealed the occurrence of fungal fruit body-specific *Sphingomonadaceae* types.

Truffles (*Tuber* spp.) are ascomycetous ectomycorrhizal fungi that form symbioses with the roots of gymnosperm and angiosperm and produce hypogeous ascocarps. Due to the organoleptic characteristics and limited distribution, some truffles are highly prized as edible fungi. Among them, the white truffle (*Tuber magnatum* Pico) is commercially the most important species. Little is known about the factors that control the truffle life cycle, such as spore germination, mycorrhizal formation, and fruit body development (Mello et al., 2006). Previous studies on

cultivated bacterial communities isolated from the mycorrhizal root tips of *Tuber borchii* revealed bacterial strains with a stimulatory or inhibitory effect on *in vitro* growth of truffle mycelium (Sbrana et al., 2002). The proceeding molecular characterization of bacterial communities from *Tuber borchii* and *Tuber magnatum* ascocarps ascertained the prevalence of α-Proteobacteria and γ-Proteobacteria among the clone library and the cultivated bacteria of both truffles, respectively (Barbieri et al., 2003, 2005). However, to date, there is no information on the occurrence and ecological consequence of sphingomonads in truffle ascocarps. In this study, we isolated a bacterial consortium from the fruit body of *Tuber magnatum*, confirmed the presence and performed functional biochemical characterization, of an isolate affiliated to the family *Sphingomonadaceae*.

MATERIALS AND METHODS

Collection and selection of bacterial strains

Bacterial isolates were collected from fresh and healthy ascocarps, carefully washed with sterile water and flame sterilized. The 100µl of PBS-Tween 20 solution (0.8g NaCl, 0.02 g KCl, 0.176 g K_2HPO_4, 0.024 g KH_2PO_4 and 200 µl Tween 20) was applied to sterile cut gleba and then inoculated on YME agar supplemented with cycloheximide (50 mg/l) and nystatin (40 mg/l). The pure bacterial isolates were preliminary tested for growth on nitrogen depleted Fiodorov medium (Rodina, 1969). The strains that grew in nitrogen-limited conditions were molecularly characterized according to the sequence of 16S rDNA. One of the strains (TMG 022C) was affiliated to the genus *Sphingobium*.

DNA isolation and PCR amplification

Bacterial genomic DNA extraction was performed using the GenElute Bacterial Genomic DNA kit (Sigma Aldrich, USA) following the manufacturer's instructions. PCR amplification of partial 16S rRNA gene was carried out in 20 µl reaction mixtures containing 2µl DNA extract, 9 µl GoTaq® Green Master Mix (Promega, USA) and 0.4 µl of 50 µM of each primer (63F 5'CAGGCCTAACACATGCAAGTC3' and 1397R 5'GGGCGGWGTGTACAAGGC3'). The following PCR program was used: 94°C for 8 min, followed by 32 cycles of (94°C for 1 min, 52°C for 1 min, 72°C for 2 min), and finally 72°C for 10 min. PCR products were purified with ExoSAP-IT kit (GE Healthcare, USA) and sequenced.

Phylogenetic analysis

To identify the bacterial isolate TMG 022C, a partial sequence of 16S rDNA was compared with published sequences on NCBI GenBank using BLASTn (Altschul et al. 1997). The 16S rDNA sequences of TMG 022C and sequences of 16S rDNA of all known *Sphingobium* species obtained from NCBI GenBank were aligned in CLUSTAL W (Thomson et al. 1997). A phylogenetic tree was constructed in MEGA 4 (Tamura et al. 2007) using the neighbor-joining method based on a pair-wise distance matrix with the Kimura two-parameter nucleotide substitution model. The topology of the tree was evaluated by the bootstrap resampling method with 5000 replicates.

Phenotypic and metabolic fingerprinting of the isolates

The pure bacterial isolate was preliminary characterized based on the cell morphology, the endospore formation, the results of the Gram staining, catalase and oxidase test, indole production and ability to hydrolyze urea according to Tindal et al. (2007).

In vitro phosphate solubilization assays

The solubilization of sparingly soluble $Ca_3(PO_4)_2$ was estimated in an NBRIP liquid culture (Nautyal, 1999). To investigate the different carbohydrate influence, commonly found in fungi in combination with a different nitrogen form, on bacterial P-solubilization, glucose was replaced with mannitol or trehalose, and $(NH_4)_2SO_4$ was replaced with KNO_3 or arginine in the original NBRIP broth, in equal amounts, respectively. Quantitative estimation of the soluble phosphate was recorded by the colorimetric phosphomolybdate method (Murphy and Riley, 1962). Final

values of soluble P were calculated from the standard curve obtained using 0-2 mg l^{-1} of KH_2PO_4.

Nitrate reduction and denitrification activity assays

Detection of nitrate dissimilation was performed as described by Roussel-Delif et al. (2005).

Determination of ammonium production

The bacterial isolate was grown in Peptonic water for 48h at 28°C. After incubation, the bacterial suspensions were centrifuged (10 000 x g for 10 min), and released ammonium in the culture supernatant was determined using Nessler's reagent. The development of yellow to brown color was assayed with a spectrophotometer at 425 nm. The amount of released ammonium was determined from a standard curve obtained using 0-4 mg l^{-1} NH_4Cl.

Nitrification activity assay

The bacterial isolate was inoculated in 6 ml of the minimal medium supplemented with $(NH_4)_2SO_4$, 0.5 g l^{-1} or $NaNO_2$, 0.5 gl^{-1}. After incubation at 28°C, the presence of nitrate and nitrite was detected as described in Roussel-Delif et al. (2005).

In vitro siderophore production assay

The ability of bacterial isolates to mobilize iron via the siderophore production was assayed on the chrome azurol S (CAS) medium (Alexander and Zuberer, 1991).

In vitro biomolecules degradation assays

To test its possible role in the degradation of cell components, the bacterial isolate was assayed for the production of extracellular proteolytic enzymes with gelatin and casein as substrates as described in Tindall et al. (2007). For detection of lecithinolytic (phospholipolytic) and lipolytic activities, egg-yolk and Tween 80 (1% w/v) according to Lanyi (1987), were used as substrates. After being selected as glucose (YME) and mannitol (Fiodorov media) consumer, the isolate was further tested for its possible role in the ascocarp life cycle by utilization of trehalose and decomposition of the fungal cell wall compounds (β-glucans and chitin), or possible decomposition of soil materials of a plant origin (cellulose, pectin). The ability of the isolate to utilize trehalose was assayed according to Dekker (1997). For testing the chitinolytic activity, the protocol described by Chernin (1995) was used. Bacterial ability to produce 1-3 β-glucanases was assayed as described by Mahasnen (1980), using curdlan instead of pachyman. The production of cellulolytic enzymes was determined after Teather and Wood, 1982. Production of pectinolytic enzymes was tested as described by Hankin et al., 1971.

RESULTS

A collection of bacterial isolates from *Tuber magnatum* ascocarp, selected on the basis of the appearance of colonies after five days incubation on 30°C in a nitrogen-depleted media with D-mannitol as a sole carbon source, included one non-slimy, yellow pigmented isolate designated as TMG022C. BLASTn comparison of the 16S rDNA gene sequence with published sequences on NCBI GenBank revealed the closest sequence similarity of the strain TMG022C to *Sphingobium sp.*

Phylogenetic analysis

The phylogenetic analysis based on the construction of the 16S rDNA gene sequence-based phylogenetic tree clearly showed that the strain TMG022 C (Ass. No. JF915707) belongs to the genus *Sphingobium*, being the most closely related to *Sphingobium amiense* YT^T (AB047364) (Fig. 1).

Phenotypic characterization

The phenotypic properties of the strain TMG022 C were similar to those of the related *Sphingobium* species (Table 1). Colonies were yellow-pigmented. The strain was Gram-negative, catalase and oxidase positive, formed rod-shaped cells and no spores. It was negative for glucose and lactose fermentation.

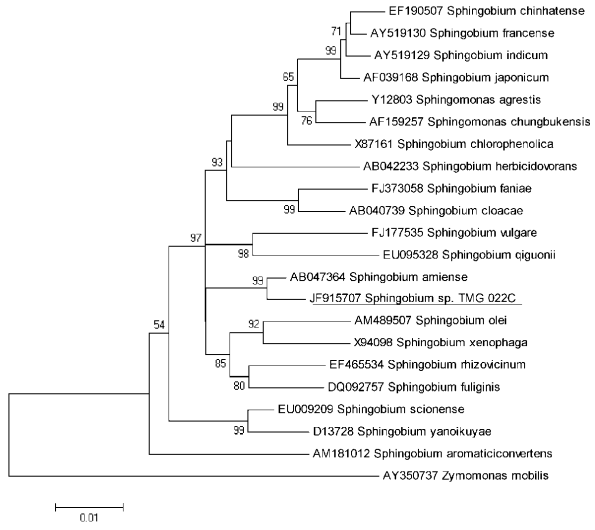

Figure 1. Neighbor-joining phylogenetic tree based on partial 16S rDNA sequences showing the evolutionary relationship between strain TMG022C and other representatives of the genus *Sphingobium*. The tree was rooted using *Zymomonas mobilis* as an outgroup. Bootstrap values (5,000 replicate runs) of at least 51% are given. Investigated strain was underlined. Scale bar = Kimura two-parameter distance

Table 1. Differential characteristics of bacterial isolate TMG022 C and its closest relatives. Strains: 1, *Sphingobium* sp. TMG022 C (this study); 2, *Sphingobium amiense* YT^T (Ushiba et al., 2003); 3, *Sphingobium yanoikuyae* JCM 7371^T (Ushiba et al., 2003); 4, *Sphingobium xenophagum* BN6^T (Stolz et al., 2000).

Characteristic	1	2	3	4
Indol	-	-	ND	ND
Citrate	-	-	+	-
D-trehalose	+	ND	ND	ND
D-mannitol	+	-	-	-
Glucose	+	+	+	+
Methyl Red	-	ND	ND	ND
Voges-Proskauer	-	ND	ND	ND
Reduction of nitrate to nitrite	+	-	-	-
Urease production	+	-	-	-

+, Positive reaction; –, negative reaction; ND, no data available.

The process of denitrification was not confirmed. The ammonium production by ammonification resulted in 269.2 ± 53.4 μg (NH_4^+) ml^{-1} of released ammonium.

In vitro inorganic phosphorus solubilization assay

The isolate *Sphingobium* sp. TMG022C solubilized $Ca_3(PO_4)_2$ in liquid NBRIP media. Experiments with different carbon sources revealed that the level of PSA in the NBRIP media by the strain *Sphingobium* sp. TMG022C was dependent on the carbon-nitrogen source combination. The highest level of solubilized phosphate was recorded in the NBRIP media with glucose or mannitol as a sole carbon source and $(NH_4)_2SO_4$ as sole nitrogen source (Table 2). In all treatments, the detected drop of pH correlated with increases in the free phosphate concentration (Table 2). Nitrate was a poor nitrogen source for PSA, while arginine could be used as a nitrogen source only when trehalose or mannitol was supplied (Table 2).

In vitro biomolecules degradation

Sphingobium TMG022 C produced chitinases, β-glucanase and lechitinase (phospholipase), while lipolytic activity was observed on both Tween 80-supplemented media and the egg-yolk agar (Table 3). The abilities to hydrolyze cellulose, pectin, casein and liquefy gelatin were not detected. The strain grew on CAS medium but without observed production of siderophore (Table 3).

DISCUSSION

Sphingomonads are metabolically versatile and able to utilize a wide range of naturally occurring organic compounds as well as many types of refractory environmental contaminants (Balkwill et al., 2006). Many strains of the family *Sphingomonadaceae* have been isolated from soils, particularly from soil contaminated by policyclic aromatic hydrocarbons (PAH), rhizosphere soil and aquatic habitats (Daane et al., 2001; Leys et al., 2004; Young et al, 2008), but their ecology still remained little known.

Table 2. Phosphate solubilization activities of *Sphingobium* sp. TMG022C in NBRIP medium on different carbon and nitrogen sources. NBRIP media with varying carbon (glucose, mannitol, trehalose) and nitrogen (NH_4^+, arginine, NO_3^-) sources were inoculated and incubated on 25 ^{0}C for 5 days. Solubilized phosphates were measured spectrophotometrically and concentrations calculated from the standard curve. pH values were measured prior to measurements of the free phosphates concentrations

Nitrogen source	Trehalose		Mannitol		Glucose	
	(µg/ml)	pH	(µg/ml)	pH	(µg/ml)	pH
$(NH_4)_2SO_4$	4.5 ± 4.1	8.44 ± 0.05	301.6 ± 18.7	5.35 ± 0.04	454.1 ± 26.7	3.76 ± 0.01
KNO_3	30.4 ± 5.1	5.31 ± 0.07	19.8 ± 3.6	5.85 ± 0.04	78.5 ± 16.4	3.85 ± 0.02
Arginine	177.6 ± 10.5	6.05 ± 0.05	149.4 ± 2.9	6.27 ± 0.03	48.8 ± 9.3	5.60 ± 0.05

Table 3. *In vitro* production of enzymes involved in utilization of fungal and plant specific carbohydrates, in degradation of general biomolecules and production of siderophores by Sphingbium sp. TMG022 C. + detected activity; - absence of activity; [a] – lipase detection on medium with the egg-yolk

Isolate	Chitinase	Pectinase	CMC - cellulase	Tween 80	Lipase [a]	Lechitinase	β-glucanase	Gellatinase	Caseinase	Siderophore
TMG022 C	+	-	-	+	+	+	+	-	-	-

In this study, we performed functional biochemical characterization of a *Sphingobium sp*., isolated from *Tuber magnatum* ascocarp and selected it as capable for growing in nitrogen-limited conditions. *Sphingobium sp*. TMG 022C degraded urea, performed ammonification and reduced nitrate to nitrite, but was not capable of supporting nitrification (Table 1). According to these features, *Sphingobium* sp. TMG 022C was clearly different from some of its closest relatives (Fig. 1, Table 1). However, dissimilatory nitrate reduction was detected for the phylogenetically close *Sphingobium fuliginis* (Prakash and Lal, 2006), for *Sphingobium rhizovicinum* (Young et al., 2008) which is grouped close to *Sphingobium sp.* TMG 022C in the phylogenetic tree (Fig. 1), as well as for distantly related *Sphingobium scionense* (Liang and Lloyd-Jones, 2010). Truffle soils are influenced by water and oxygen fluctuations during the year, when the soil becomes dry and oxygenated or waterlogged (microaerobic conditions). Microbial metabolism-driven processes, such as ammonification and nitrate reduction, could be very important for the truffle mycelia nitrogen supply; *Sphingobium sp.* TMG022C, supported by fungus-derived carbohydrates, could take an active role in these processes.

The strain *Sphingobium sp*. TMG022 C successfully solubilized tricalcium phosphate with the highest efficiency while utilizing mannitol and glucose as a carbon source, and ammonium sulfate as the nitrogen source (Table 2). Previous investigation revealed the predominance of mannitol in the hyphae of *Tuber borchii* mycelium (Ceccaroli et al., 2003), and the observed high efficiency of phosphate solubilization while *Sphingobium sp.* was mannitol-fed could imply fungus-bacteria metabolic interaction. The observed pH decrease that accompanied tricalcium-phosphate solubilization (Table 2) is a mechanism well-known for the mineral phosphate solubilization employed by many microorganisms (Rodríguez et al., 2006). In *Tuber magnatum* a calcareous type of soils, where the phosphates are often

fixed in insoluble forms, the role of truffle-associated bacteria in phosphorous nutrition, supporting fruit body development, can be assumed. Barry et al. (1995) reported the capacity of the tufts of hyphae that sprout from the scales of the peridium of the ascocarps of *Tuber aestivum* and *Tuber melanosporum* to absorb phosphate. Their findings provide additional evidence for nutritional autonomy of fruit bodies as suggested by Barry (1994), and additionally demonstrated rapid phosphate metabolism by the ascocarp. Murat et al. (2005) suggested that *Tuber magnatum* invests more in forming fruiting bodies that in root colonization and that there is not direct linkage between mycorrhizas and fruiting bodies. Results presented here (Table 1, Table 2) suggest that the bacterial communities associated with *Tuber magnatum* ascomata, including *Sphingobium sp.*, could contribute to mycelium nutrition throughout the fruit body development.

On the other hand, *Sphingobium sp.* TMG 022C had the ability to hydrolyze lipid molecules and the ability to degrade β-glucans and chitin, constitutive elements of the fungal cell wall, but failed to decompose the plant materials cellulose and pectin (Table 3). These features indicate an additional role of *Sphingobium sp.* in truffle ascocarp decomposition as well as facilitation of ascus opening and ascospore dispersion at the end the fructification phase.

In conclusion, the results, presented in this study for the first time, provide evidence of sphingomonad occurrence inside the truffle fruit body, as well as more detailed biochemical properties of the isolated *Sphingobium sp.* strain. The strain was capable of adapting to diverse ecological conditions such as nitrogen limitation, nitrogen biotransformation according to the environmental redox conditions, inorganic phosphate solubilization with different carbon and nitrogen sources, as well as utilization of fungi-derived carbohydrates D-mannitol and D-trehalose, β-glucans and chitin. All these properties suggest that *Sphingobium sp.* strain TMG022C could improve *Tuber magnatum* mycelium nutrition and therefore stimulate mycelium growth, or take on a role in ascocarp decomposition. Further investigation is necessary to provide a closer characterization of this strain and its relation to truffle mycelia.

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